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Effect of Polyamines on Adventitious Root Formation from Tobacco (*Nicotiana tabaccum*) Leaf Segments

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To elucidate the effect of polyamines on adventitious root formation, we investigated the relationship between the frequency of adventitious root formation and the endogenous content of free polyamines in tobacco leaf segments which had been treated with polyamine biosynthesis inhibitors and polyamines. Adventitious root formation was inhibited in rooting medium (10 μM IAA) with methylglyoxal-bis(guanylhydrazone) (MGBG) or cyclohexylamine (CHA), and promoted with spermidine and putrescine. Treatment with high IAA (100 μM) medium plus CHA or MGBG promoted rooting up reversion of the rooting inhibition than the one treated with high IAA concentration alone. Spermidine promoted adventitious root numbers on low IAA (1 μM) medium when applied during culture period. The rooting inductive phase (in the presence of IAA) was determined by periodical transfer of leaf segments from IAA-containing medium to IAA free medium, and by changing polyamine contents, to be inductive phase. Putrescine and spermidine were accumulated to a maximum during the inductive phase. Therefore, the results point out the involvement of polyamines in inductive phase of adventitious root formation in tobacco leaf segments.

Keywords: adventitious root, spermidine, spermine, MGBG, CHA

The polyamines have considerable influence on the growth and differentiation of plant cells and tissues. More recently, it has been suggested that polyamines may play an important regulatory role in the process of organogenesis (Bagni *et al.*, 1980; Serafini Fracassini *et al.*, 1984; Evans *et al.*, 1988). Polyamines present in animals, microorganisms and plants (Bagni and Torigiani, 1992) are involved in adventitious buds formation (Tiburcio *et al.*, 1988; Tanimoto *et al.*, 1994) and adventitious root formation(Biondi *et al.*, 1990; Hausman *et al.*, 1994).

Regarding the biosynthesis of polyamines in plants, it is well established that arginine and ornithine may contribute to putrescine (PUT) biosynthesis via the action of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) (Evans and Malmberg, 1989). In either case, spermidine (SPD) and spermine (SPM) biosynthesis are started from putrescine by the addition of aminopropyl residues derived from S-adenosylmethionine with the help of S-adenosylmethionine decarboxylase (SAMDC) (Smith, 1985). Furthermore, it is established polyamine inhibitors by such as α-difluromethylornithine (DFMO), α-difluromethylarginine (DFMA), cyclohexylamine (CHA), dicyclohexylamine (DCHA) and methylglyoxal-bis(guanylhydrazone) (MGBG). DFMO (Metcalf *et al.*, 1987), DFMA (Kalio *et al.*, 1981), CHA (Evans and Malmberg, 1989) and DCHA (Biondi *et al.*, 1990) have been shown to inhibit specifically ODC, ADC and spermidine synthase activity, respectively. Thus, putrescine and spermidine synthesis is inhibited. MGBG also inhibit SAMDC activity, and then spermidine and spermine synthesis are inhibited (Alhonen-hongisto *et al.*, 1980). We are available for studies polyamine function on plant organogenesis if it is regulated artificially endogenous polyamine levels by exogenous polyamine inhibitors and polyamines. Thus, these attempts have been partially studied on the formation of adventitious buds (Tanimoto *et al.*, 1994) and adventitious roots (Martin-Tanguy *et al.*, 1991), respectively. However, the role of polyamines in organogenesis such as adventitious bud and root formation in the same plant is not well understood.

In this study, the treatment of low level (1 μM IAA) or high level (100 μM IAA) experiments than adventitious root formation condition (10 μM IAA)

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was not formed adventitious root. This is not certain whether lack or abundance of polyamine level according to hormone level changes. Hence, we further investigated that the effects of changes in IAA and polyamine levels on adventitious root formation, and used tobacco leaf segments, a well-known formation of adventitious bud and root.

MATERIAS AND METHODS

Plant Material and Data Interpretation

Tobacco (*Nicotiana tabacum*) line NT1 seeds were germinated aseptically. Leaf explants (3 cm long), were obtained by removing the petioles, margins, and mid veins from young leaves. Leaf segments were incubated at 25±1°C in the dark for 20 days on media. The basal medium used for rooting was Murashige & Skoog's (1962) medium. The number of adventitious roots from the explants was counted on more than 20 leaf segments and the data presented show average number of adventitious roots per leaf segment. Experiments were repeated 3 times and standard errors were calculated in each case.

Effect of CHA and MGBG on Adventitious Root Formation

To investigate the effect of CHA and MGBG on adventitious root formation, tobacco leaf segments were incubated on MS-solidified medium containing 10 μM IAA (rooting medium) plus CHA and MGBG, and 100 μM IAA plus CHA and MGBG.

Effect of Polyamines on Adventitious Formation

To investigate the effect of polyamines on adventitious root formation, tobacco leaf segments were incubated on MS-solidified medium containing 10 μM IAA and 1 μM IAA plus several different concentration of polyamines such as putrescine, spermidine and spermine.

Determination of the Inductive Phase of Rooting

To determine relationship between rooting inductive phase and periods of hormone treatment, leaf segments were incubated for 1-6 days on MS medium containing IAA 10 μM, and then transfered to MS hormone-free medium, for 14-19 days (total incubation period was 20 days for each leaf segments). After 20 days, adventitious root formation was investigated.

Investigation of the Changes in Endogenous Polyamine Levels

To investigate the effect of polyamines and polyamine inhibitors during adventitious root formation, leaf segments were incubated for 5 days on MS medium containing 1 μM IAA, 100 μM IAA, and 100 μM IAA plus 1 μM MGBG which showed reversion of the rooting inhibition. Then the levels of endogenous polyamines in the segments were analyzed.

Polyamine Extraction and Estimation

Polyamines were measured according to the modified procedure of Goren *et al.* (1982). Samples (500 mg) were homogenized in 0.5 mL 5% ice-cold perchloric acid (PCA). The homogenate was centrifuged at 12,000 g for 30 min at 4°C, and the supernatant fraction was used for polyamine analysis. Polyamines were separated using TLC precoated plates of Silicagel (Sigma) in a chloroform:triethylamine (25:2, v/v) solvent system. Spots visualised under UV were scraped off and eluted in 4 mL ethylacetate. The fluorescence was measured using electronic photofluorimeter (excitation: 350 nm, emission: 500 nm), and the results were compared with dansylated standards.

RESULTS

Effect of CHA and MGBG on Adventitious Root Formation

The adventitious root formation on leaf segments was inhibited in treatments with 10 μM IAA medium contained CHA and MGBG (Fig. 1A) than the control. In treatments with 0.1 μM CHA or 1000 μM MGBG the adventitious root formation was reduced to 76% and 86% less than the control. In treatments with 100 μM IAA the adventitious root formation was inhibited, but and adventitious root formation was generally increased in treatment of 100 μM with CHA or MGBG (Fig. 1B). In treatments with 100 μM CHA the adventitious root formation was increased to 181% of the 100 μM IAA medium, and then in 1 μM MGBG was increased to 272%, but reduced less than the treatment of 10 μM IAA alone.

Effect of Polyamines on Adventitious Root Formation

In treatments of 10 μM IAA (Fig. 1C) with 0.1 μM or 1000 μM spermidine the adventitious root formation was increased to 22% and 40%, but with 100 μM or 1000 μM putrescine the adventitious root formation was increased to 53% and 40%. In 10 μM IAA medium with 10 μM spermine, the adventitious root formation was decreased to 225%. In all treatments of 1 μM IAA and polyamines (Fig. 1D) the adventitious root formation was promoted, in 1 μM putrescine or 10 μM spermine the adventitious root formation was promoted by 54% than 1 μM IAA alone, and in 1000 μM spermidine was increased by 218% than 1 μM IAA, but decresed less than the treatment of 10 μM IAA alone.

Adventitious Rooting Induction Phase

When leaf segments were incubated on rooting medium (MS medium containing 10 μM IAA) for different times ranging from 0-5 day before transferring them to hormone frec medium a period of 5 days was sufficient to induce 96% rooting (Fig. 3). But adventious roots were induced for 1 day on rooting medium.

Changes of Polyamine Levels

The endogenous putrescine levels were increased significantly during early culture period in all treatments, and later they varied depending on the treatment. In the control (Fig. 4A) putrescine levels were increased significantly during the first culture day, and spermidine and spermine levels were increased gradually after first culture day. In 1 μM IAA treatment (Fig. 4B), the number of roots was few, all polyamine levels were similar to the control, but putrescine and spermidine levels were lower than the control. In 100 μM IAA treatment (Fig. 4C), the number of roots was lower than the control, putrescine levels were increased significantly during the first culture day, but lower than the control, spermidine levels were increased during the third culture days, and also showed a similar profile to the control. In 100 μM IAA plus 1 μM MGBG (Fig. 4D) the adventitious root formation considerably reversed. Putrescine levels reached maximum at the third culture days, while in the control maximum level was observed at the first day. Spermidine and spermine levels were similar to the control.

DISCUSSION

Because the polyamines were involved in plant organogenesis, artificial regulation of endogenous polyamine levels by applying exogenous polyamines and/or polyamine inhibitors will provide insights on the function of polyamines in plant organogenesis.

MGBG inhibited adventitious root formation in mung bean hypocotyl cuttings (Jarvis *et al.*, 1983). This study also indicates that adventitious root formation inhibited more strongly by the application of MGBG than the CHA (Fig. 1A). Hausman *et al.* (1994) reported that polyamine application promoted adventitious root formation in hazel micro-shoots. Our data indicate that adventitious root formation is improved or inhibited depending on the concentration of putrescine and spermidine in the rooting medium containing 10 μM IAA. However, spermine inhibited the rooting regardless of the applied concentration (Fig. 1C). These results suggest that adventitious root formation might be controlled by putrescine and spermidine. MGBG strongly inhibited adventitious root formation as concentration is gradually increased, whereas CHA remarkably inhibited adventitious root formation either at higher or lower

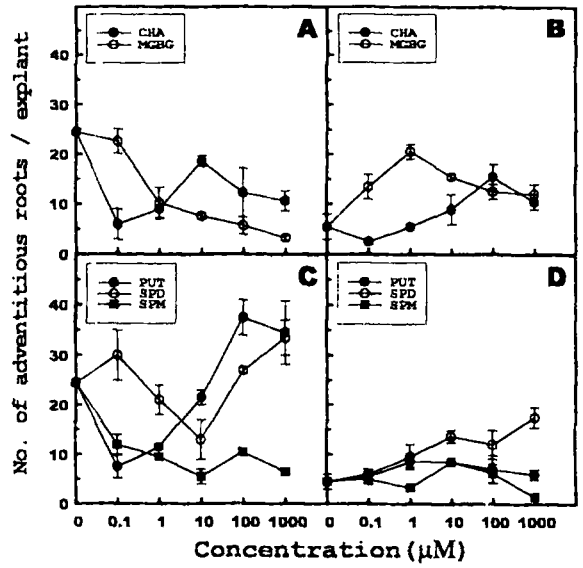

Fig. 1. Effect of polyamine inhibitors and polyamines on adventitious root formation in IAA-containing media on tobacco leaf segments after 20 days of culture. A, 10 μM IAA+polyamine inhibitors; B, 100 μM IAA+polyamine inhibitors; C, 10 μM IAA+polyamines; D, 1 μM IAA+ Polyamines. Vertical bars indicated standard errors.

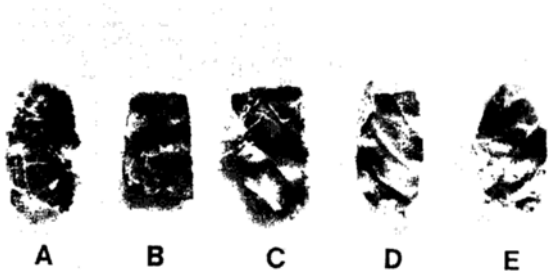

Fig. 2. Effect of IAA, MGBG and spermidine on adventitious root formation from tobacco leaf segments after 20 days of incubation. A, Control (10 μM IAA); B, 100 μM IAA; C, 100 μM IAA+1 μM MGBG; D, 1 M IAA; E, 1 M IAA+1000 μM Spermidine.

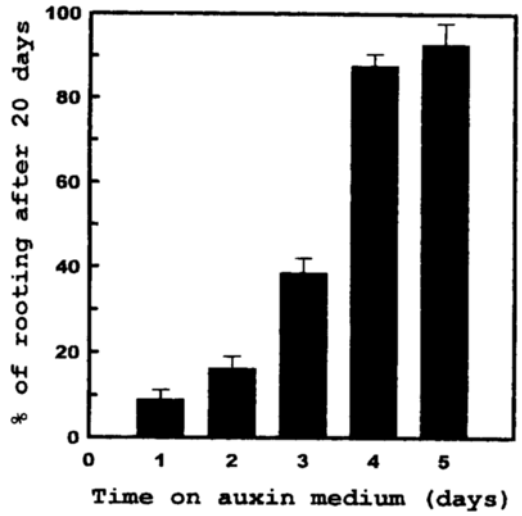

Fig. 3. Rooting percentage of tobacco leaf segments after 20 days on IAA free medium preceded by different times on 10 μM IAA medium. Vertical bars indicateed standard errors.

concentration rather than at the medium concetration of 10 μM (Fig. 1A, Fig. 2A). Since MGBG, the specific inhibitor of biosynthesis of spermidine and spermine, inhibited adventitious root formation better than CHA, a specific inhibitor of spermidine synthase which converts putrescine into spermidine, it can be suggested that spermidine and spermine are evidently related to the adventitious root formation.

Treatment with 100 μM IAA also (Fig. 1B, Fig. 2B) reduced adventitious root formation compared with the control. This indicates that 100 μM IAA treated segments accumulated too much levels of polyamines in the tissue. For this reason CHA and MGBG were treated to reduce polyamine levels. As a result adventitious root formation was increased more in 100 μM IAA with polyamine inhibitors than in 100 μM IAA alone (Fig. 1C, Fig. 2C). This suggests that MGBG and CHA reduce endogenous polyamine levels which has been increased by high level of IAA, and promote adventitious root formation. Adventitious root formation was also reduced at lower level of IAA (Fig. 1D, Fig. 2D) because of low level of endogenous polyamines. All treatments increased adventitious root formation (Fig. 1D, Fig. 2E) when various concentrations of polyamines were treated together with 1 μM IAA. These results suggest that reduction of polyamine levels by lower level of IAA is reversed by exogenous application of polyamines. These results are consistent with earlier observations that the addition of spermidine in tobacco thin layers (Torrigiani *et al.*, 1993) and spermine in soybean cotyledons (Han *et al.*, 1994) resulted in a significant reversion of the rooting inhibition which was caused by the CHA or MGBG treatment. Since 1000 μM exogenous spermidine application increased adventitious root formation upto 218% of the treatment of 1 μM IAA alone, it seems that in tobacco leaf segments is more strongly affected by spermidine than spermine.

Whether adventitious root formation are effected by the control of endogenous polyamine levels or not, polyamine levels are determined during adventitious rooting induction phase (Fig. 3). As a result, leaf segments treated with 1 μM IAA showed lower putrescine level than 10 μM IAA (Fig. 4B), whereas the treatments of 100 μM IAA showed lower putrescine levels than the control, unchanged spermine levels, and higher spermidine levels (Fig. 4C). These suggest that leaf segments of low level IAA decrease polyamine levels, whereas high level of IAA changed putrescine into spermidine, or not spermidine into spermine. Thus, we thought IAA activated spermidine synthase. Also leaf segments treated with 100 μM IAA plus 1 μM MGBG (Fig. 4D) caused a considerable reversion of the inhibition of adventitious root formation. The reason for this reversion is due to recovery of putrescine and spermidine levels to the control level, and especially, due to inhibitory action of MGBG on spermidine synthase. Putrescine levels reached maximum at the third culture day, spermidine at the fourth, and these are related to the result that the number of adventitious root showed lower than the control. Therefore putrescine and spermidine levels are involved in adventitious root formation. But when segments treated with 1 μM IAA plus polyamines showed adventitious root formation, whereas sper-

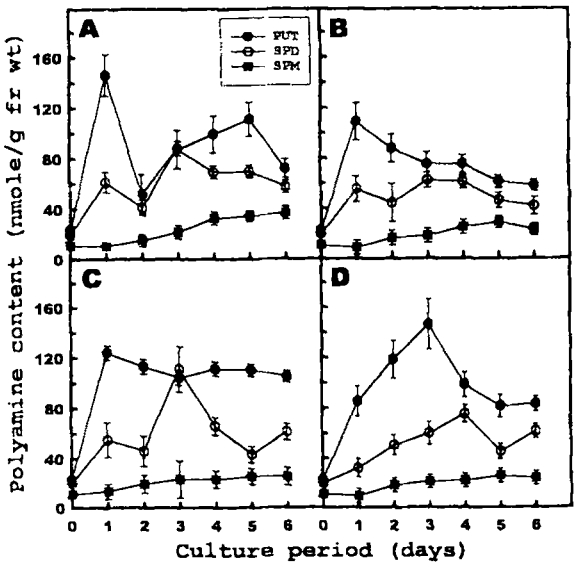

Fig. 4. Changes in free polyamine titers during adventitious root formation periods. A, Control (10 μM IAA); B, 1 μM IAA; C, 100 μM IAA; D, 100 μM IAA+1 μM MGBG. Vertical bars indicateed standard errors.

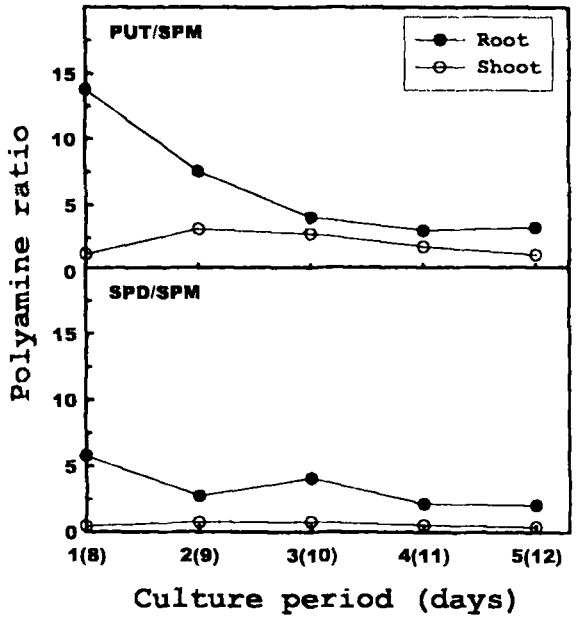

Fig. 5. Ratios of PUT/SPM and SPD/SPM during adventitious root formation on tobacco leaf segments. PUT, Putrescine; SPD, Spermidine; SPM, Spermine. Numbers in brackets are the day of shoot induction.

mine promoted or inhibited as contents adventitious root formation depending on the applied concentrations (Fig. 1D). These suggested effects of polyamines on adventitious root formation is not related with a single polyamine.

During the adventitious root formation spermine levels were low and unchanged, but spermidine levels were highly variable (Fig. 4). These are contrasted to the results of Han *et al.*, (1996) in which spermidine levels were not changed, while spermine levels increased in shoot formation of tobacco leaf segments. This suggests that adventitious root formation has something to do with spermidine while shoot formation has to do with spermine. But polyamine level changes alone can not explain organogenesis. Experiments in the control of different polyamine levels using polyamine inhibitors with polyamines (Fig. 1B, 1D) resulted that adventitious root formation in tobacco leaf segments involved spermidine deeply, but in soybean cotyledonary segments were involved spermine (Han and Jo, 1994), Torenia stem segments (Tanimoto *et al.*, 1994), and popula leaf segments (Kim *et al.*, 1993) reported spermidine promoted shoot formation. As a result the formation of shoots and roots is not affected by the a particular polyamine alone. It was reported that polyamine ratio were related to culture period in organogenesis (Santos *et al.*, 1993; Schwartz *et al.*, 1986), these studies showed that PUT/SPM and SPD/SPM ratio (Fig. 5) in adventitious root formation were higher and increased than in shoot formation (Han *et al.*, 1996). It was expected that especialy SPD/SPM ratio was important in organogenesis because in tobacco leaf segments SPD/SPM ratio was higher, and shoot formation reversion. These results might indicate that spermidine and SPD/SPM ratio are deeply involved in adventitious root formation of tobacco leaf segments.

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LITERRATURE CITED

Alhonen-Hongisto L., P. Seppanen and J. Janne. 1980. Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal-bis(guanylhydrazone). *Biochem. J.* **192**: 941-945.

Bagni, N., B. Malucelli and P. Torrigiani. 1980. Polyamine storage substances and abscisic acid-like inhibitors during dormancy and very early activation of *Helian thustuberosus. Physiol. Plant.* **49**: 341-345.

Bagni N. and P. Torrigiani. 1992. Polyamines: a new class of growth substances. In progress in plant growth regulation, karssen C.M., Van Loon L.C. and Vreugdenhil D., ed., Klumer Academic Pub., Dordrecht, The Netherland, 264-275.

Biondi, S., T. Diaz, I. Iglesias, G. Gamberini and N. Bagni. 1990. Polyamines and ethylene in relation to adventitious root formation in *Prunus avium* shoot cultures. *Physiol. Plant.* **78**: 474-483.

Evans, P.T., B.L. Holaway and R.L. Malmberg. 1988. Biochemical differentiation in the tobacco flower probed with monoclonal antibodies. *Planta* **175**: 259-269.

Evens, P.T. and R.L. Malmberg. 1989. Do polyamines have roles in plant development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**: 235-269.

Goren R, N. Palavan, H. Flores and A.W. Galston. 1982. Changes in polyamine titer in etiolated pea seedlings following red-light treatment. *Plant Cell Physiol.* **23**: 19-26.

Han, T.J., D.W. Lee and S.H. Lee. 1994. Effects of polyamine inhibitors and polyamines adventitious root formation from soybean cotyledons. *Korean J. Plant Tissue Culture.* **21**: 106-110.

Han, T.J. and H.I. Jo. 1994. Effects of cyclohexylamine and polymaines on the adventitious root formation from soybean cotyledons. Korean *J. Plant Tissue Culture.* **21**: 207-213.

Han, T.J. M.G. Jeon and H.I. Jo. 1996. Effect of polyamines on adventitious shoot formation on tobacco (Nicotiana tabaccum) leaf segments. *Korean J. Plant Tissue Culture* **23**: 45-50.

Hausman, J.F., C. Kevers and T. Gasper. 1994. Involvement of putrescine in the inductive rooting phase of poplar shoots raised *in vitro. Physiol. Plant.* **92**: 201-206.

Jarvis B.C, P, R.M. Shannon and S. Yasmin. 1983. Involvement of polyamines with adventitious root development in stem cuttings of mung bean. *Plant Cell Physiol.* **24**: 677-683.

Kallio A., P. McCann and P. Bey. 1981. DL-α (difluoromethyl) arginine: a potent enzyme-activated inhibitor of bacterial arginine decarboxylase. *Biochemistry* **20**: 3163-3166.

Kim S.H, M.W Kim, Y.H. Kang and S.H. Lee. 1993 Correlations between polyamines and adventitious shoot regeneration from *Populus* leaf segments. *Korean J. Bot.* **36**: 1-8.

Martin-Tanguy, J., D. Tepfer and D. Burtin. 1991. Effects of Ri TL-DNA from *Agrobacterium rhizogenes* and the inhibitors of polyamine synthesis on growth, floral development, sexual organogenesis and polyamine metabolism in tobacco. *Plant Science.* **80**: 131-144.

Metcalf B., P. Bey, C. Dauzin, J. Jung, P. Casara and J. Vevert. 1987 Catalytic irreversible inhibition of mammalian ornithine decarboxylase (EC 4.1.1.17) by substrate and product analogues. *J. Am. Chem. Soc.* **100**: 2551-2553.

Murashige, T. and F. Skoog. 1962. A revises medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-497.

Santos, M., I. Claparols and J.M. Torne. 1993. Effect of exogenous arginine, ornithine, methionine and r-amino butyric acid on maize (*Zea mays* L.) embryogenesis and polyamine content. *J. Plant Physiol.* **142**: 74-80.

Schwartz M, A. Alltman, Y. Cohen and T. Arzee. 1986. Localization of ornithine decarboxylase and changes in polyamine content in root meristems of *Zea mays. Physiol. Plant.* **62**: 485-492.

Serafini-Fracassini D., P. Torrigiani and C. Branca. 1984. Polyamines bound to nucleic acid during dormancy and activation of tuber cells of *Helianthus tuberosus. Physiol. Plant.* **60**: 351-357.

Smith, T.A. 1985. Polyamines. Annu. Rev. Plant Physiol. and Plant Mol. Biol. 36: 117-143. Serafini-Fracassini, D., P. Torrigiani and C. Branca. 1984. Polyamines bound to nucleic acid during dormancy and activation of tuber cells of *Helianthus tuberosus. Physiol. Plant.* **60**: 351-357.

Tanimoto, S., Y. Matsubara and N. Ishioka. 1994. Significance of spermidine in the initiation of adventitious buds in stem segments of *Torenia. Plant Cell Physiol.* **35**: 1071-1077.

Tiburcio, A.F., R. Kaur-Sawhney and A.W. Galston. 1988. Polyamine biosynthesis during vegetative and floral bud differentiation in thin layer tobacco tissue cultures. *Plant cell Physiol.* **29**: 1241-1249.

Torrigiani, P., M.M. Altmura, S. Scaramagli, F. Capitani, G. Falasca and N. Bagini. 1993. Regulation of rhizogenesis by polyamines in tobacco thin layers. *J. Plant Physiol.* **142**: 81-87.

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